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Published in:
Biocatalysis and Biotransformation

DOI:
10.1080/10242420410001710056

Biocatalysis and Biotransformation, 2004 VOL. 22 (2). pp. 139–144

Taylor & Francis
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Novel Biocatalysts by Identification and Design

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Enzymes produced from bacteria and eukaryotic organisms are presently being used for a large variety of different biotechnological applications. The rapidly increasing demand for enzymes which are active towards novel and often non-natural substrates has triggered the development of novel molecular biological methods of enzyme isolation and design. The metagenome approach is a cultivation-independent method which allows the direct cloning and expression of environmental DNA thereby providing access to a wealth of so-far unknown biocatalysts. Additionally, newly identified or existing biocatalysts can be further optimized by different methods of directed evolution. Here, the principle of the metagenome approach is outlined and a strategy is presented for the optimization of a bacterial lipase using a combination of rational design and directed evolution.

Keywords: Bacillus subtilis lipase; biodiversity; directed evolution; metagenome

INTRODUCTION

More than five thousand years ago, fermentation was discovered as a process for the production of alcohol. Also, without having any knowledge of the existence of enzymes and microorganisms, the Egyptian civilisation used yeast for baking bread, a technique which later became known as whole cell biocatalysis (Liese *et al.*, 2000). A breakthrough for using enzymes to catalyze chemical reactions occured in 1858, when Louis Pasteur succeeded in separating the (+) and (−) enantiomers of racemic tartaric acid using the fungus *Penicillium glaucum*. This experiment constitutes the first successful biocatalytic kinetic resolution (Pasteur, 1858).

Nowadays, biocatalysis using whole cells, crude cell extracts or purified enzymes has achieved a position of steadily increasing importance for the biotechnological production of food additives, agrochemicals, cosmetics and flavours, and, in particular, for pharmaceuticals. The rapidly growing demand for these compounds results in a pressing need to identify biocatalysts with novel and desired properties. Therefore, extended programs aim to collect novel microorganisms, plants or animals from all over the world to use them as a source for the identification of novel enzymes. However, natural evolution has adjusted today's enzymes to perfectly fit into their respective physiological niches. As a consequence, their biochemical properties like stability, activity, and enantioselectivity normally do not fulfill the needs of a chemical process.

In order to overcome these difficulties, a repertoire of tools for enzyme engineering was developed with most of them operating in a rational way. Firstly, the 3D-structure of a given enzyme is solved to allow the identification of important amino acids. Then, enzyme variants are constructed by classical site-directed mutagenesis based on predictions derived from the analysis of the 3D-structure and finally, these variants are biochemically characterized. More recently, a set of new methods was developed which are summarized as "*in vitro*" or "directed" evolution and provide a powerful tool for the creation of novel biocatalysts whithout requiring any knowledge of the enzyme structure or its catalytic mechanism (Cherry and Fidantsef, 2003; Farinas *et al.*, 2001; Jaeger *et al.*, 2001; Powell *et al.*, 2001; Tao and Cornish, 2002).

In this article we will describe promising novel approaches to (i) identify biocatalyst genes and (ii) optimize an enzyme by directed evolution.

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ISSN 1024-2422 print/ISSN 1029-2446 online
DOI: 10.1080/10242420410001710056

THE METAGENOME APPROACH FOR MINING NEW BIOCATALYST GENES

Starting about 3.8 billion years ago, natural evolution has created prokaryotes capable of coping with adverse living conditions and inhabiting almost every ecological niche by metabolizing virtually all known substrates. Therefore, it is not surprising that prokaryotes became the dominant form of life and may represent the largest constituent of total biomass on earth (Whitman *et al.*, 1998). Despite their abundance, current estimates indicate that more than 99% of the prokaryotes present in natural environments like soil, water, sediments, or plant surfaces are not readily culturable in the laboratory by standard techniques and therefore remain inaccessible for biotechnological applications (Amann *et al.*, 1995). This fact is illustrated by the observation that one gram of soil may contain thousands of different species, however, only about 10–100 different species will be caught by known cultivation methods (Torsvik *et al.*, 2002). Obviously, these uncultivated bacteria not only significantly contribute to the ecology of a bacterial community (Torsvik and Ovreas, 2002) but they also represent an enormous biotechnological potential (Cowan, 2000). The so-called metagenome approach (Handelsman *et al.*, 1998) can overcome the cultivation problem by direct isolation and cloning of environmental DNA (eDNA) resulting in metagenome libraries which represent the genomes of all microorganisms present in a given sample independent of their culturability. The microbial diversity present in such a library can be analysed by 16S-rRNA sequencing and these libraries also constitute the starting material to identify novel biocatalyst-encoding genes by using high-throughput screening or selection methods (Fig. 1).

Nevertheless, several problems exist in constructing metagenome libraries. Isolated eDNA derived from soil-samples may be contaminated with phenolic compounds or humic acids which inhibit the following cloning steps, namely the digestion catalyzed by restriction endonucleases, the ligation, or the eDNA-amplification using the polymerase chain reaction (PCR). Several promising approaches were described to circumvent these problems (Rochelle, 2001). Different isolation strategies are suitable to recover the spectrum of prokaryotic diversity present in a respective sample: (1) The *ex situ* methods in which cells are isolated and concentrated from soil prior to their lysis, and (2) the *in situ* methods in which cells are lysed directly within the soil material (Courtois *et al.*, 2001). (3) A third strategy uses the enrichment of microorganisms for a desired enzyme activity prior to the isolation of metagenomic DNA. This strategy proved successful for isolating complete biotin operons and many different biocatalyst-encoding genes (Borneman, 1999; Entcheva *et al.*, 2001; Radajewski *et al.*, 2000; Schmeisser *et al.*, 2003; Voget *et al.*, 2003). (4) A new and elegant approach starts with an amplification of specific partial gene sequences using conserved and degenerate oligonucleotides called metagenome sequence tags (MST's). Subsequently, shuffling of the cloned fragments and PCR-amplification generates biocatalyst genes of increased diversity as shown for dehalogenases and haloperoxidases (Lorenz *et al.*, 2002). Undoubtedly, the metagenome approach will quickly generate an enormous amount of novel enzyme genes, however, novel tools have to be developed to increase the efficiency of cloning and expression.

DIRECTED EVOLUTION TO ENGINEER NOVEL BIOCATALYSTS

Many different enzymes have been subjected to optimization by directed evolution including proteases, amylases, laccases, phytases, and cellulases (Cherry and Fidantsef, 2003). Substrate specificity, thermal stability, and organic solvent resistance, but also more difficult properties such as cofactor-independence or enantioselectivity were evolved (Funke *et al.*, 2003; Lingen *et al.*, 2003; May *et al.*, 2000; Moore and Arnold, 1996; Wong *et al.*, 2004, Zhao and Arnold, 1999). In our group, a variety of different directed evolution methods have been used to evolve lipases which represent the most important class of enzymes for organic chemistry (Jaeger and Eggert, 2002; Jaeger and Reetz, 1998). In particular, we have extensively characterized the bacterial lipases from *Pseudomonas aeruginosa* and *Bacillus subtilis* and have studied their structure-function relationships by site-directed (Eggert *et al.*, 2000; Liebeton *et al.*, 2001) and random mutagenesis methods (Funke *et al.*, 2003; Liebeton *et al.*, 2000; Reetz *et al.*, 1997).

Lipases have been studied for many years, but there is still a debate over a general definition for a lipase and whether structural features can be identified in lipases which govern the lipase reaction or determine its specificity (Chahinian *et al.*, 2002b; Verger, 1997). In contrast to esterases, lipases show almost no activity as long as the substrate is present in the monomeric state. However, when the solubility limit of the substrate is exceeded and an emulsion is formed, a sharp increase in lipase activity occurs, a phenomenon termed interfacial activation (Sarda and Desnuelle, 1958). The first lipase 3D structures which were solved for human pancreatic and a fungal lipase (Brady *et al.*, 1990; Winkler *et al.*, 1990) provided an elegant explanation for this phenomenon. Both lipases were found to

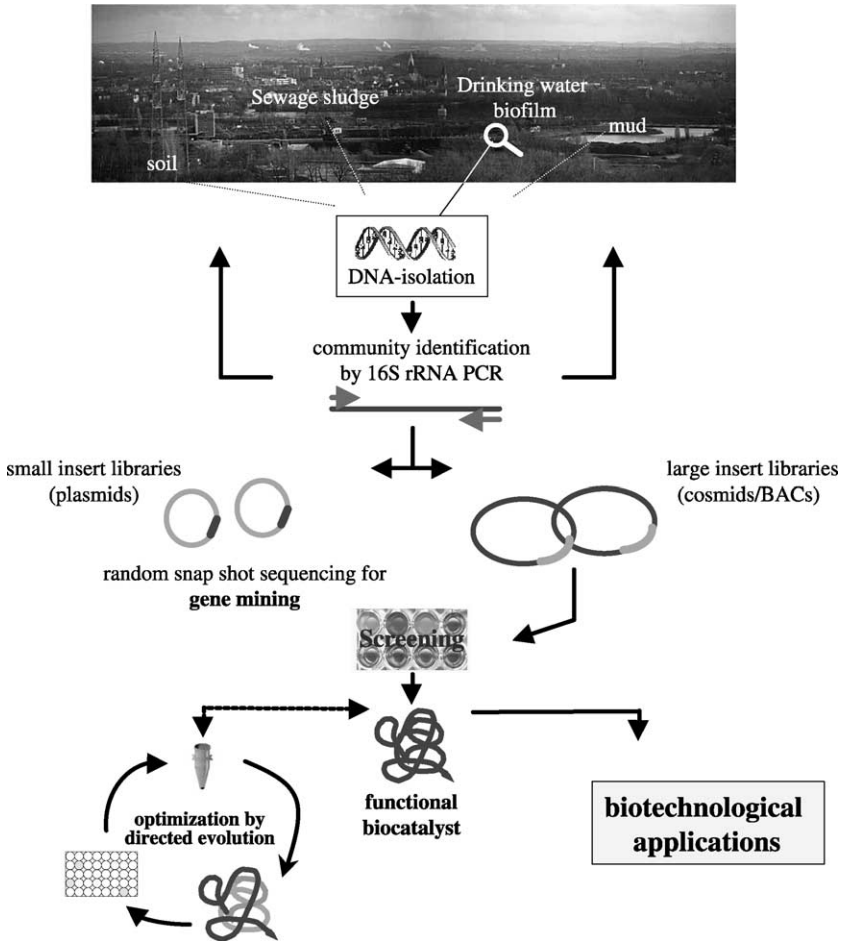

FIGURE 1 Schematic outline of the metagenome approach to isolate novel biocatalysts. DNA representing the collective genomes of a given habitat is PCR-amplified for prospecting biodiversity by 16S rRNA sequencing and cloned into appropriate vectors used for identification by DNA sequencing, expression cloning, and subsequent determination of enzyme activities, e.g. by high-throughput screening, the novel biocatalysts may further be optimized by directed evolution.

possess α-helical polypeptide chains capable of covering the active site thereby making the catalytic amino acid residues inaccessible to substrate molecules. The α-helices which were called ''lid''-structures would move upon contact with the substrate interface resulting in conformational reorientations which render the active site residues accessible for the substrate. This ''lid-hypothesis'' was later confirmed by elucidating the X-ray structures of several other inhibitor-bound lipases (Brzozowski *et al.*, 1991; Nardini *et al.*, 2000) and has since been used to discriminate between 'true' lipases and esterases (Jaeger *et al.*, 1999; Verger, 1997). In addition, the lid-domain of lipases has also been identified as being important for substrate recognition, catalytic activity, substrate specificity, (Carriere *et al.*, 1998; Chahinian *et al.*, 2002a; Brocca *et al.*, 2003), enantioselectivity (Liebeton *et al.*, 2000), and activity in organic solvents (Fishman and Cogan, 2003; Mingarro *et al.*, 1995). In summary, lid-like structural elements constitute the most important structural elements of lipases.

During the last few years, three-dimensional structures of 26 lipases, including 8 lipases from bacterial origin, have been elucidated (Table I). Surprisingly, not all of these structures revealed the presence of a lid-domain. The lipases from *Bacillus subtilis* (Dartois *et al.*, 1992; Eggert *et al.*, 2000; Lesuisse *et al.*, 1993) are catalytically active on long chain lipids although they do not possess a lid-domain. *B. subtilis* lipase A (BSLA) is the smallest lipase (181 amino acids; 19.3 kDa) known so far which has a solvent-exposed active site located at the bottom of a small cleft between two loops consisting of residues 10–15 and 131–137. The BSLA active site contains a preformed oxyanion hole so that conformational changes are not required to ensure the formation of the transition state. Thus, its small size and the absence of a lid make BSLA the minimal α/β-hydrolase-fold enzyme (Fig. 2) (van Pouderoyen *et al.*, 2001). Therefore, we have chosen this enzyme as a starting point to engineer by directed evolution new lid structures on top of the existing minimal α/β-hydrolase core.

TABLE I Overview of solved three-dimensional structures of lipases. Lipases lacking a lid are marked with an asterisk.

Eucaryotic	
Mammals/pancreatic lipases	*Homo sapiens* (human)
	Equus caballus (horse)
	Cavia porcellus (guinea pig)
	Sus scrofa (pig)
	Bos taurus (cattle)
	Canis familiaris (dog)
	Rattus norvegicus (rat)
Mammals/gastric lipases	*Homo sapiens* (human)
	Canis familiaris (dog)
Fungi	*Rhizomucor* miehei
	Geotrichum candidum
	**Fusarium solani*
	Candida rugosa
	Penicillium camembertii
	Rhizopus delemar
	Thermomyces lanuginosa
	Candida antarctica
	Rhizopus niveus
Procaryotic	
Gram-negative	*Burkholderia glumae*
	Chromobacterium viscosum
	Burkholderia cepacia
	Pseudomonas aeruginosa
Gram-positive	*Streptomyces exfoliatus*
	**Bacillus subtilis*
	Bacillus stearothermophilus L1
	Bacillus stearothermophilus P1

Construction of *B. subtilis* Lipase with an Artificial Lid-domain

In computer-based studies the lipolytic enzymes cutinase from *Fusarium solani pisi*, acetylxylanesterase from *Penicillium purpurogenum*, and human pancreatic lipase were compared with respect to the domains located close to their active sites. These enzymes were chosen because their three-dimensional structures show a high homology to the structure of BSLA. Furthermore, lids or lid-like-domains were present in all three X-ray structures but are missing in the *Bacillus* lipase. Subsequently, these lids were modeled into the structure of BSLA indicating experimental options to engineer these lids into BSLA without disturbing the core α/β-hydrolase fold (Fig. 3).

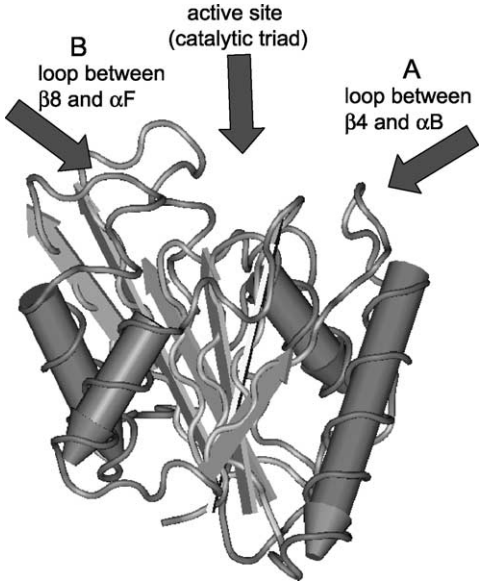

FIGURE 2 Crystal structure of *B. subtilis* lipase A (BSLA). The positions of catalytic triad residues Ser77, His156 and Asp133 are indicated in purple, and the two loop regions surrounding the active site are labelled with A (β4 and αB) and B (β8 and αF), respectively.

BSLA-variants Carrying an Artificial Lid-domain Show Hydrolytic Activity

The lid-sequences were engineered into the BSLA-gene by using a modified two-step megaprimer PCR method. The resulting BSLA-variants (Fig. 3) were overexpressed in *E. coli* and tested for enzymatic activity with the substrates *p*-nitrophenyl palmitate (spectrophotometric assay, Fig. 4A) and tributyrin (agar plate assay, Fig. 4B). All variants showed enzymatic activity, at least against one of the substrates, although the variants containing artificial lid domains exhibited a lower activity than the wild-type. Therefore, we are currently trying to optimize the artificial lid domains by subjecting the corresponding DNA-fragments to random mutagenesis using ep-PCR. Preliminary results with a high-throughput screening assay on tributyrin indicator plates indicated that we have created several new variants which exhibit wild-type activities.

OUTLOOK

Enzyme-based biocatalysis provides a means to carry out chemical processes efficiently and economically. This fact is increasingly recognized as reflected by a rapidly growing enzyme market which was valued at approximately $1.5 billion already in 2000 (Cherry and Fidantsef, 2003) and is expected to increase by an average annual growth rate of at least 10%. The future success of enzyme technology will depend on the development of efficient and cost-effective processes for the production and downstream processing of enzymes. Even more important will be the identification of novel enzyme genes from natural sources, their high-level and functional expression, as well as their optimization for desired properties by directed evolution. The combination of these methods will undoubtedly result in a major breakthrough for enzyme technology, also in entirely new areas of technical applications.

Acknowledgements

This work was supported by the German Bundesministerium für Bildung und Forschung (Competence Network "Genome Research on Bacteria for the Analysis of Biodiversity and Its Further Use for the Development of New Production Pro-

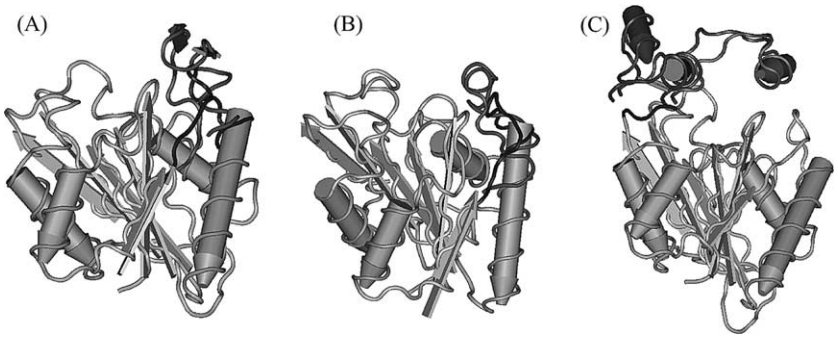

FIGURE 3 Structural models of BSLA-variants carrying artificial lid-domains. Lids engineered into BSLA at the given amino acid positions were from (A) acetylxylanesterase at position 39–51, (B) cutinase at position 39–51 and (C) human pancreatic lipase at position 153–155. The artificial lid domains are shown in red; the human pancreatic lipase lid is modeled both in the closed (red) and in the open (purple) conformation. The positions of the catalytic triad residues Ser77, His156, and Asp133 are indicated in purple.

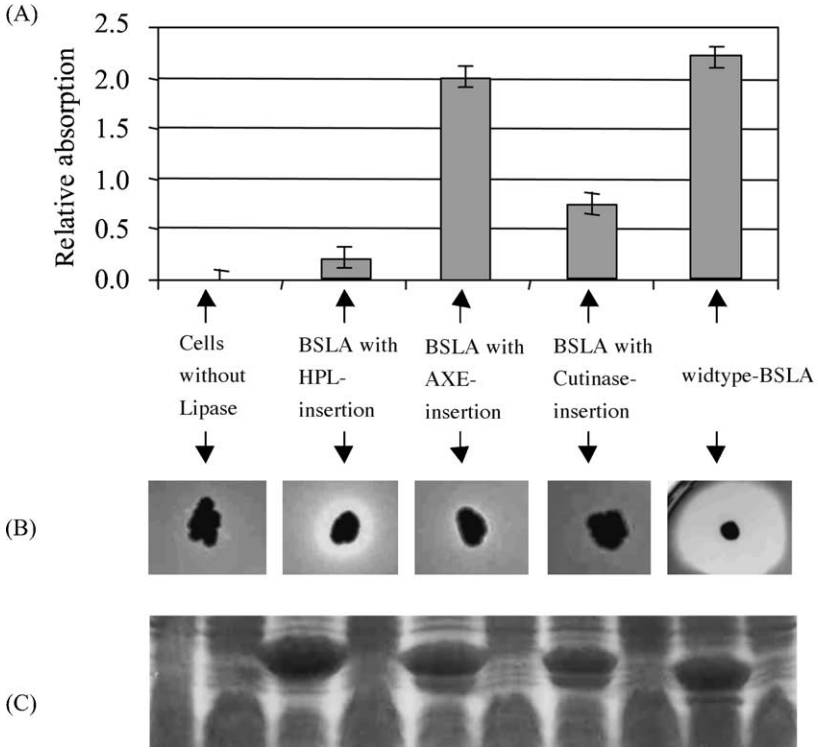

FIGURE 4 Catalytic activity of newly constructed BSLA-variants carrying artificial lid domains obtained from human pancreatic lipase (HPL), acetylxylanesterase (AXE) and cutinase. The catalytic activity towards (A) *p*-nitrophenyl palmitate and (B) tributyrin were tested in a spectrophotometric (Eggert *et al.*, 2000) and an indicator plate assay, respectively. (C) Protein overexpression was detected by SDS-polyacrylamide gel electrophoresis of whole cell-lysates obtained from *E. coli* overexpressing the BSLA variants and subsequent staining with Coomassie Brilliant Blue. The lanes show cell extracts isolated before (right lanes) and after induction of lipase gene expression by addition of 0.3 mM isopropyl-β-D-thio-galactopyranoside (left lanes).

cesses'') and by the European Commission in the framework of the program Biotechnology (project no.:BIO4-CT98—0249). KEJ wishes to thank Manfred T. Reetz and his group at the Max-Planck-Institut für Kohlenforschung, Muelheim an der Ruhr, Germany, for an exciting and fruitful collaboration in the field of creating enantioselective enzymes by directed evolution.

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